- RNA isolation phase (w/ RNEasy Plus Mini Kit):
    - Every step at room temperature
    - Gather sample (cell, organoid) (< 1x10^7 cells/sample).
    - Lyse directly or trypsinized and collected as a cell pellet by adding 400 uL Buffer RLT Plus and keep it in 1.5 mL tube
      If organoid, use P1000 pipette tip to break up organoid and do a swirling motion "beating an egg."
    - Homogenize the lysate by vortexing for 1 min
      >> Pause point: -20^{o}c or -80^{o}c for several months
    - Transfer lysate to purple gDNA Eliminator Spin Column. Pipette up and down until foamy.
    - Centrifuge for 30s at 8000g. Save Flow-Through (/FT). Discard column.
      *All centrifuge at 20-25*c (not cool below 20*c)
    - Add 350 uL of 70% EtOH to FT. Mix and immediately transfer 700 uL of FT to the pink RNEasy Spin Column.
    - Close lid & Centrifuge for 15s at 8000g. Discard FT. Save column.
    - Add 700 uL Buffer RW1 to the same pink column.
    - Close lid & Centrifuge for 15s at 8000g. Discard FT. Save column.
    - Add 500 uL Buffer RPE to the same pink column.
    - Close lid & Centrifuge for 15s at 8000g. Discard FT. Save column.
    - Add 500 uL Buffer RPE to the same pink column.
    - Close lid & Centrifuge for 2min at 8000g. Discard FT. Save column.
    - Dry column for 2min.
    - Place pink column in a new 1.5-mL Eppendorf tube for elution.
    - Add 35-60 uL dw directly onto the membrane
    - Close lid & Centrifuge for 1min at 8000g to elute RNA.
    - Measure [RNA] with NanoDrop.
    - Store RNA at 4 C until further use.

- RT phase:
    - Dilute RNA with dw in PCR-tube strip.
    - Make RT Master Mix.
    - Distribute RT MM into diluted RNA in PCR-tube strip.
    - On PCR machine, incubate at 42 C for 2 hours then 4 C.
    - Store cDNA RT product at 4 C until further use.
    - Formula for RNA dilution per sample:
        - Ideally, target mass of RNA = 1000 ng (max2000 ng)
        - If smallest [RNA] = 65 ng/uL, then set target mass at 65 ng/uL x 15 uL = 1000 ng
        - Calculate volume of RNA needed to reach target mass for each sample.
        - Top off with dw to reach total volume of 15 uL
        - Total volume for RNA diluted = 15 uL
    - Formula for RT Master Mix in 2-mL Eppendorf tube:
        - Multiply each volume below by (the number of reactions needed + 10% for pipetting error)

        - 2 uL 10X RT Buffer (Applied Biosystem)
        - 2 uL 10X RT Random Primers
        - 1 uL RT Enzyme (MultiScribe ReverseTranscriptase)
        - 0.5 uL dNTP
        - Total volume for RT MM per sample = 5.5 uL
    - Total volume for RT product per sample = 20.5 uL
    - Thermocycler setup for reverse transcriptase (two-step)

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

- 5RT with BioRad's iScript 5x MM
    - Dilute RNA to 1000ng or 1500 ng in PCR strip → Total Volume 8uL
    - Add 2uL 5x MM to make it to 10uL

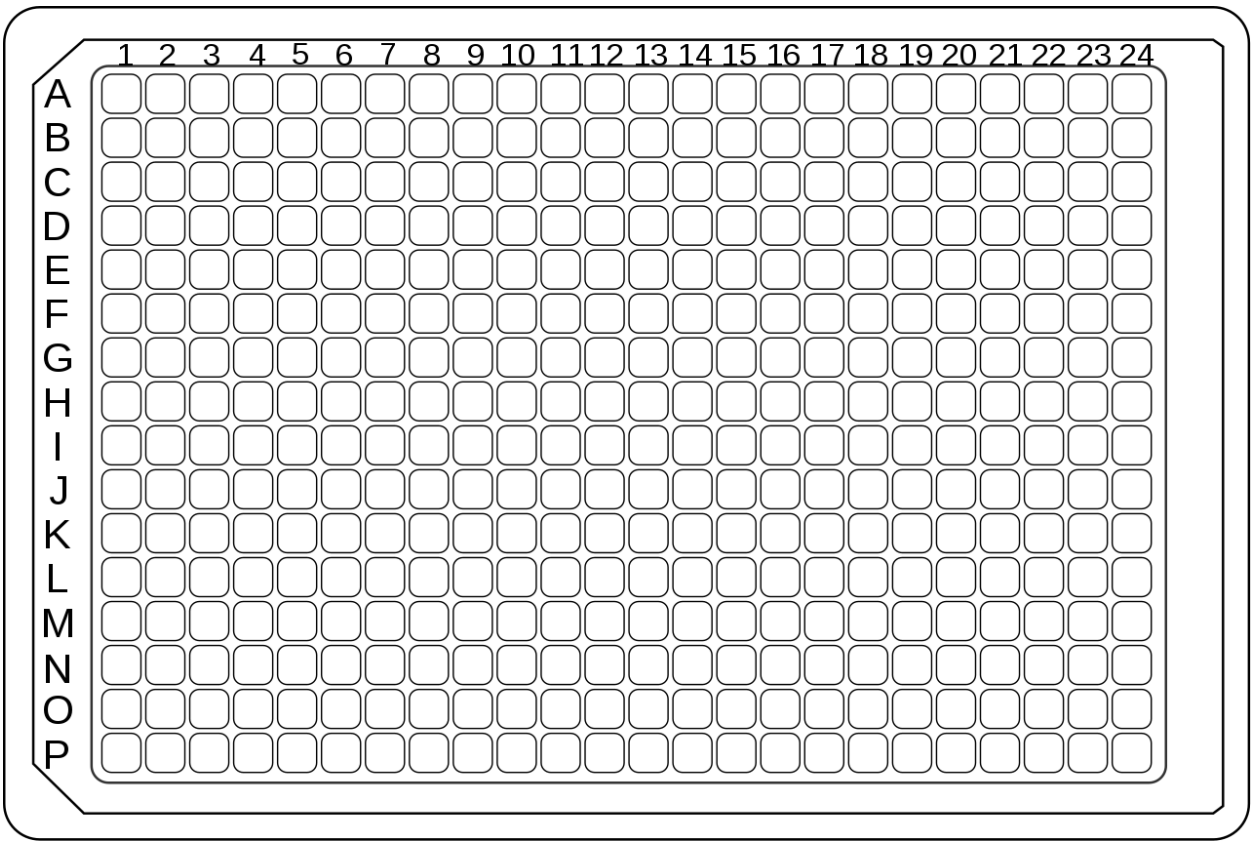
- qPCR phase (ddCT):
    - Formula for each well:
        - 5 uL 2X PowerSYBR Green MM
        - 0.2 uL 10uM primer mix
        - 0.2 uL RT product/template
        - 4.6 uL dw
    - <u>Step 1</u>: Plate Planning
        - Suppose: 5 samples, 10 genes
        - Mixtures: SYBR MM + Template; dw + primer (not waste more SYBR!)
        - Prepare 5 tubes in strip for 5 templates (dist. horizontally)
            - Each tube has enough for 10 -> 15 rxns
        - Prepare 10 tubes in strip for 10 genes (dist. vertically)
            - Each tube has enough for 5 -> 10 rxns
    - <u>Step 2</u>: Volume Planning
        - SYBR MM + Template Mixture:
            - SYBR MM: 5 uL x 15 rxns = 75 uL
            - Tempalte: 0.2 uL x 15 rxns = 3 uL
            - Total = 78 uL per PCR tube
            - Distributing horizontally 5.2 uL per well
        - Dw + Primer Mixture
            - Primers: 0.2 uL x 10 rxns = 2 uL
            - Dw: 4.6 uL x 10 rxns = 46 uL
            - Total = 48 uL per PCR tube
            - Distributing vertically 4.8 uL per well
    - <u>Step 3</u>: Doing it!
        - Distribute with the electronic multi-channel pipette
        - Each tip has a max volume of 30 uL

- qPCR phase (Nilay 2021.8.24) :
    - Make a standard
        - Tube A = Pool 3 uL cDNA from each condition to make a most concentrated standard
          e.g. 6 conditions -> 3x6 = 18 uL
        - Tube B = Dilute tube A in dw to make enough standard volume for each gene
          e.g. 10 genes -> dilute to the final volume at least 10x4.5 uL = 45 uL
        - Serial dilution of Tube B in 1:5 manner 4 times to make tube C, D, E

	Relative cDNA
Tube B	1:1 (125x)
Tube C	1:5(25x)
Tube D	1:25(5x)
Tube E	1:125(1x)

    - Formula for each rxn (10 uL/rxn)
        - 5 uL 2X PowerSYBR Green MM
        - 0.2 uL 10uM primer mix (each)
        - 2 uL 10-fold dilution RT product/template
        - 2.8 uL dw
    - Plate planning
        - Each plate has to have 1) house keeping gene 2) standard reaction for each gene
    - Thermocycler (Bio-Rad CFX)

Activation of enzyme	95c	10 min	Activate AmpliTaq Gold DNA polymerase
Denature	95c	15 sec	40x
Annealing&Elongation	60c	60 sec	
Plate readout			
Melt curve analysis	55c	0.5c increment every 5 sec	
	90c		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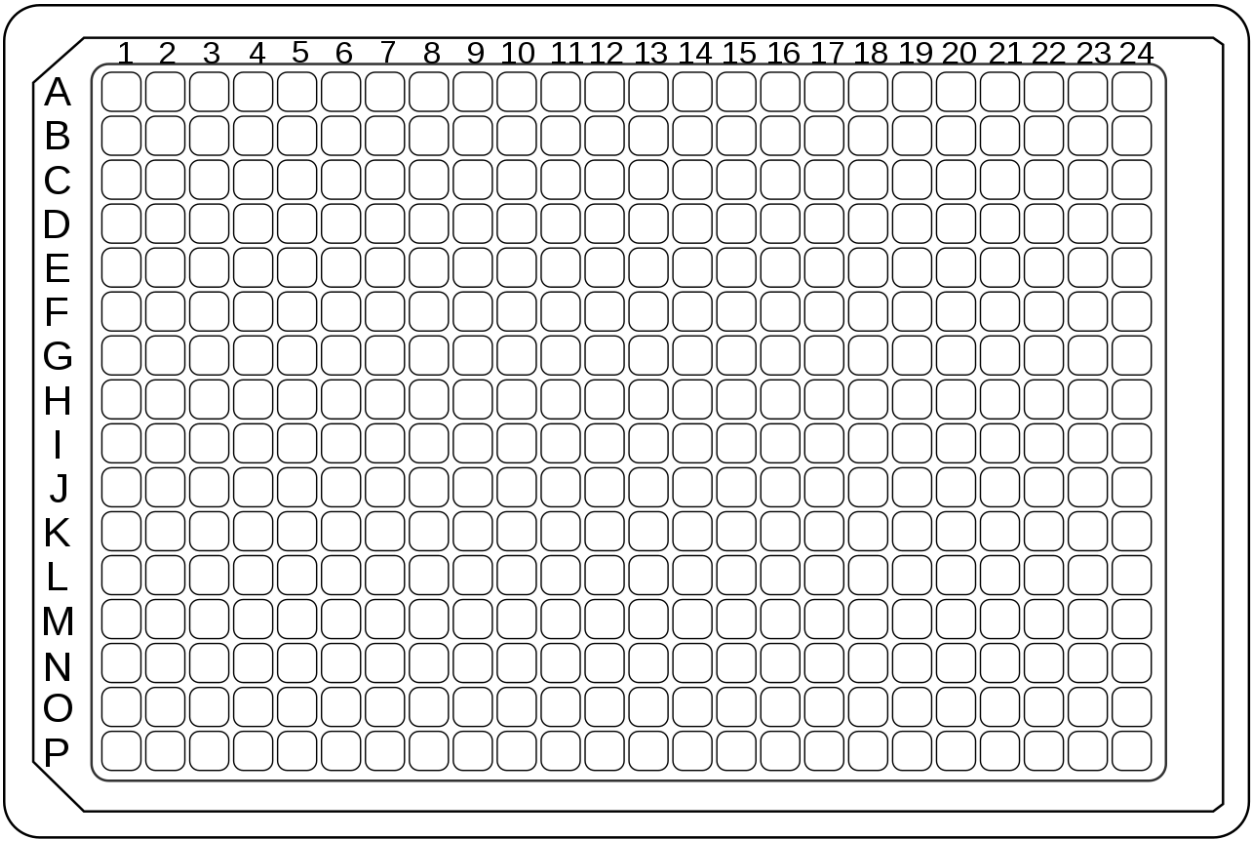
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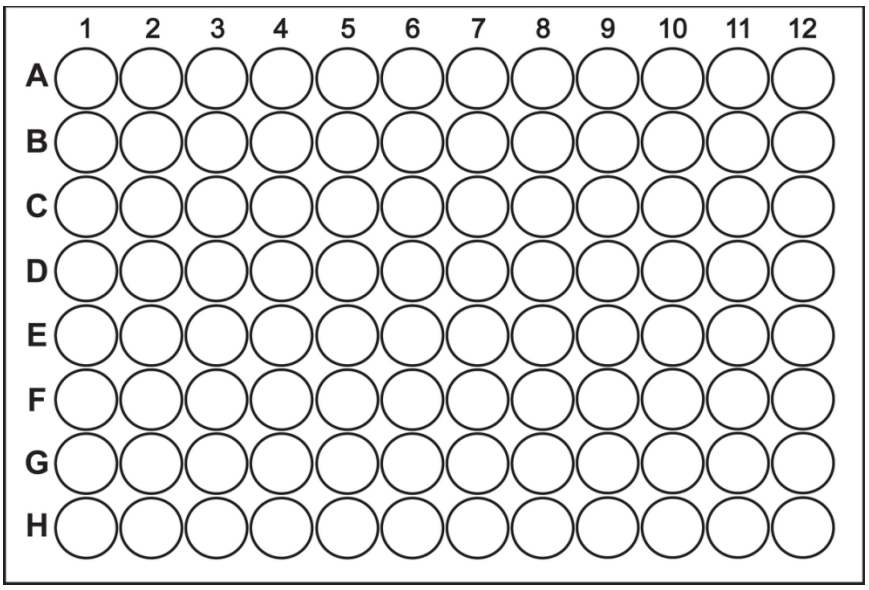
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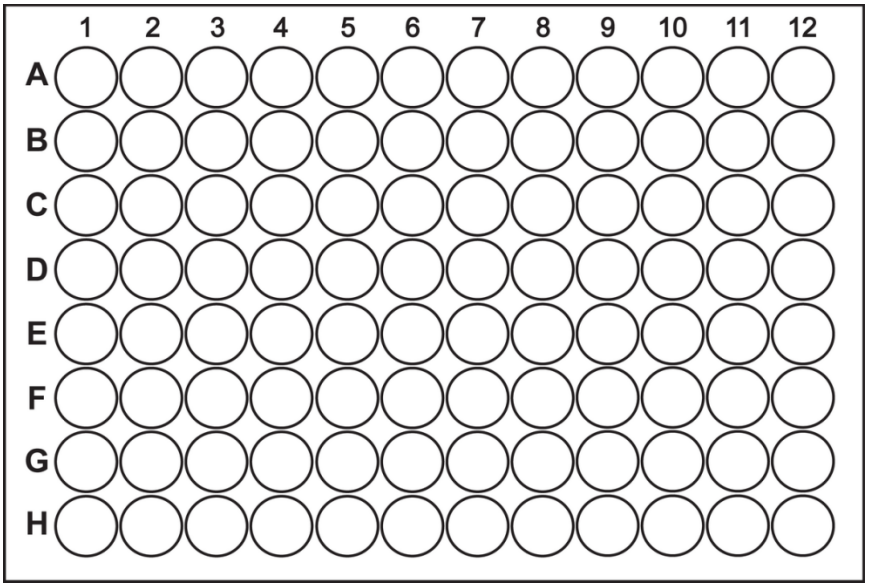
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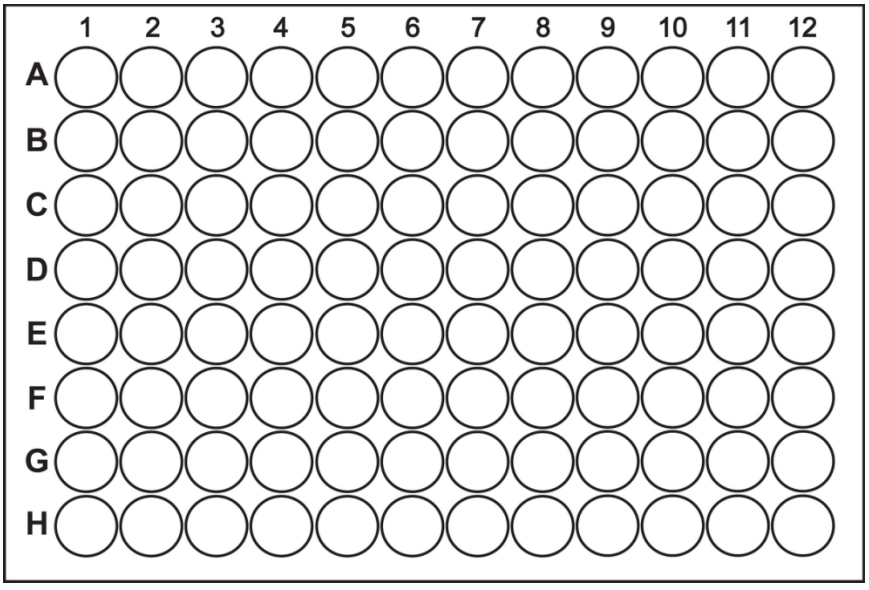
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